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Mutagenic/Carcinogenic Potential of DEHP and MEHP

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The mutagenic/carcinogenic activities of DEHP and MEHP were studied in bacteria and mammalian cells. MEHP but not DEHP exerted a dose-dependent DNA damaging effect to *B. subtilis* in Rec-assay. DEHP and MEHP showed mutagenic activities to *S. typhimurium* TA-100, with and without S-9 mix, respectively. MEHP produced not only the mutation in *E. coli* WP2 B/r but also sister chromatid exchanges (SCE) in Chinese hamster V79 cells. It also induced 8AG/6TG-resistant gene mutations and chromosomal aberrations in the V79 cells.

Transplacental administration of DEHP or MEHP to the Syrian golden hamster embryos was carried out by administering DEHP or MEHP to gravid animals on day 11 of gestation, followed by the cultivation of embryonic cells for 15-20 days. Both DEHP and MEHP induced 8AG/6TG-resistant mutation, chromosomal aberrations and morphological transformation in the embryonic cells of the Syrian golden hamster.

Introduction

The mutagenic activity of di(2-ethylhexyl) phthalate, (DEHP) was first reported in dominant lethal experiments of mice by Singh et al. in 1974 (*1*). Since then, several reports concerning the genotoxicity of DEHP on microorganisms and mammalian cells have appeared, though most of the studies failed to demonstrate its genotoxic activities (*2-4*). We have previously presented some evidence that mono(2-ethylhexyl) phthalate (MEHP), a major metabolic intermediate of DEHP in mammals, induced not only embryo/fetotoxic effects on mice but also DNA damaging/mutagenic activity to *Bacillus subtilis* (Rec-assay), *Salmonella typhimurium* TA 100 (reversion plate assay) and *Escherichia coli* WP2 B/r try$^-$ (spot test) (*5, 6*).

The present investigation was undertaken to determine whether DEHP and MEHP are mutagenic/carcinogenic in animal cells. The mutagenic/carcinogenic effects of DEHP were examined by using V79 Chinese hamster cells *in vitro* and also by using the embryonic cells of the Syrian golden hamster which were exposed to DEHP transplacentally.

This is the summary of mutagenic/carcinogenic activities of DEHP and MEHP on microorganisms and on hamster cells.

Experimental

Materials

All chemicals, biochemicals and medium constituents were purchased from Wako Pure Chemicals (Tokyo), Tokyo Kasei Chemicals (Tokyo), Sigma Chemical Co. (St. Louis, Mo.), Difco Lab. (Detroit, Mich), or Gibco (Grand Island, N.Y.). All chemicals and biochemicals were of analytical grade. MEHP was synthesized according to the method of Kenyon (*7*) and was more than 99% pure as determined by ECD-GLC analysis. Plastic dishes and flasks for tissue culture were obtained from Falcon Plastic (Oxnard, Calif.).

Assay with bacteria

All chemicals were dissolved in dimethyl sulfoxide (DMSO) for testing.

Tester strains of *S. typhimurium* TA 100, *E. coli* WP2 B/r and *B. subtilis* H17, M45 were provided by T. Kada (The National Institute of Genetics) and T. Matsushima (Institute of Medical Sci., Univ. of Tokyo).

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DNA-damaging test was performed according to Kada's Rec-assay (*8*). The Ames test was conducted by the preincubation method (*9*).

The procedure for the quantitative mutagenicity test was as follows. The bacterial cell suspensions (4×10^9 cells/ml for *E. coli* B/r WP2 and 1×10^9 cells/ml for *S. typhimurium* TA 100) in Dulbecco's phosphate buffered saline were incubated with the MEHP solution for 20 min. After the incubation, the number of revertant colonies was counted. The cytotoxicity was determined by ordinary methods.

Assay with V79 Chinese hamster cells

Mitomycin C (MMC) and ethyl methanesulfonate (EMS) were dissolved in culture medium directly and added to the cells within 30 min. In these cytogenetic studies, the general experimental methods of Ishidate (*3*), Kuroki (*10*) and Perry (*11*) were used.

Assay with Syrian golden hamster

The experimental scheme for *in vivo*/*in vitro* combination assay of the mutation induced by the administration of DEHP/MEHP is as depicted in Figure 1. The application of DEHP and MEHP to Syrian golden hamster, the preparation of primary culture and chromosome, the selection of the mutants and the assay of morphological transformation were all conducted according to the procedure described by Inui (*12*).

Results

Table 1 represents the DNA damaging potential of DEHP and its related compounds in the Rec-assay with *B. subtilis* according to the method of Kada et al. (*8*). The compounds tested were DEHP, MEHP, phthalic acid and 2-ethylhexanol. Differences larger than 1.5 mm in the inhibited zones between H-17 (*rec*$^+$) and M-45 (*rec*$^-$) were observed when 400 or 500 μg of MEHP/disk was used. No differences were observed between the inhibition length of two strains when 500 μg of DEHP or phthalic acid/disk was applied. A slight difference in the inhibitions between the two strains was observed when 500 μg of 2-ethylhexanol/disk was applied. A positive result was obtained when DEHP preincubated with mouse pancreas homogenate was used.

Table 2 shows the mutagenic potential of DEHP and MEHP in the plate assay (*9*) with *S. typhimurium* TA 100 in the presence or absence of S-9 mix. DMSO was used as the control vehicle. Both 4-nitroquinoline-1-oxide (4-NQO) and benzo(a)pyrene were used as reference compounds possessing positive effects. A level of 5 mg of DEHP/plate gave 142 induced revertant colonies/plate with S9 mix, while 1.25 mg of MEHP/plate gave 30 induced revertants/plate without S9 mix. It should be noted, however, that 1.25 mg of MEHP/plate gave no induced revertants in the presence of S9 mix. These results suggest that MEHP itself is an acting principle for the development of mutagenicity and that further metabolism of DEHP by S9 mix would lead to cessation of its activity.

The mutagenic activities of various doses of MEHP to *S. typhimurium* TA 100 and *E. coli* WP2 B/r are shown in Figure 1. The surviving cells of both bacteria decreased with the increased concentration of MEHP, and the resultant numbers of histidine-or tryptophan-independent mutant colonies/surviving bacteria cells increased sharply in a dose dependent manner.

Further studies were performed on MEHP and DEHP with the use of V79 Chinese hamster cells and the embryonic cells of Syrian golden hamster.

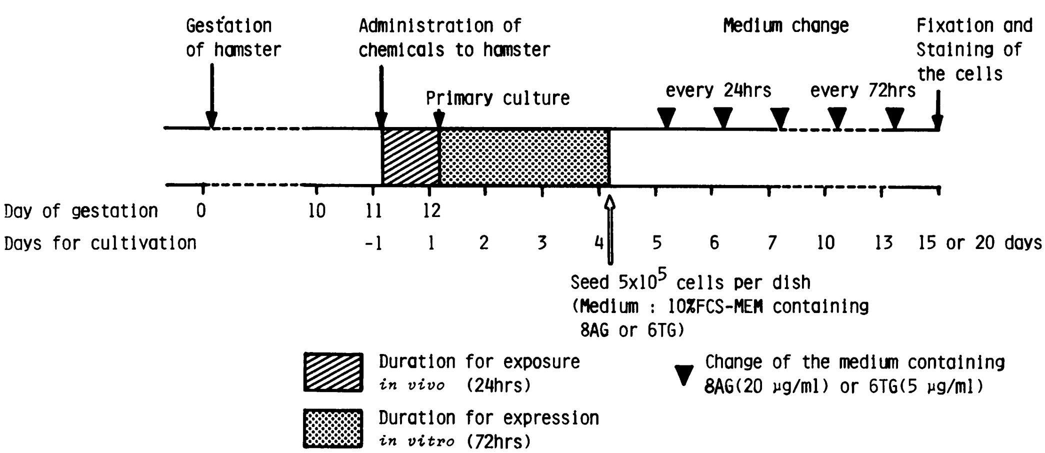

FIGURE 1. *In vivo*/*in vitro* combination assay procedure with the embryonic cells of Syrian golden hamster

Table 1. DNA-damaging potentials of DEHP and related compounds in Rec-assay with *B. subtilis*.

Compounds tested	Concn, μg/disk	Inhibition zone, mm H17 (Rec^+)	M45 (Rec^-)	Difference, (mm)	Evaluation
DEHP	500	0	0	0	−
MEHP	50	0	0	0	−
	100	0	0.5	0.5	−
	200	0	0.8	0.8	−
	300	0	1.3	1.3	−
	400	1.3	4.5	3.2	+
	500	1.0	6.0	5.0	+
Phthalic acid	500	0	0	0	−
2-Ethylhexanol	500	0.4	1.7	1.3	−

Table 2. Mutagenic potentials of DEHF and MEHP in the plate assay with or without S-9 mix by *S. typhimurium* TA-100.

Compounds tested	Amount plate	Without S9 mix[a] Revertant colonies per plate	Without S9 mix[a] Induced revertants per plate	With S9 mix[a] Revertant colonies per plate	With S9 mix[a] Induced revertants per plate
DMSO	100 μg	141 ± 7	—	182 ± 22	—
4NQO[a]	0.4 μg	264 ± 22[c]	123		
Benzo(a)pyrene	5 μg			380 ± 50[c]	198
DEHP	5 mg			324 ± 16[c]	142
MEHP	1.25 mg	171 ± 8[b]	30	171 ± 31	0

[a]All experiments were performed with five plates per group.
[b]Significantly different from controls, $p < 0.05$.
[c]Significantly different from controls, $p < 0.01$.

Table 3. Sister chromatid exchanges induced by MEHP in Chinese hamster cells.

Compounds tested[a]	Concentration, μg/ml	Time, hr	Total cells counted	Total SCEs	Mean SCEs per cell ± SD	Mean SCEs per chromosome ± SD
BrdUrd[b]	2	24	21	105	5.0 ± 1.0	0.24 ± 0.06
MMC[c]	6.7×10^{-3}	24	20	590	29.5 ± 2.2[d]	1.35 ± 0.29[d]
MEHP	25	24	20	146	7.3 ± 2.3[d]	0.36 ± 0.14[e]
	50	24	20	210	10.5 ± 3.4[d]	0.52 ± 0.17[d]
MMC	13.4×10^{-3}	3	27	237	8.8 ± 4.7[e]	0.42 ± 0.25[e]
MEHP	750	3	21	132	6.3 ± 2.6	0.29 ± 0.09
	1000	3	20	124	6.2 ± 2.7	0.30 ± 0.14
	1500	3	20	158	7.9 ± 3.7[e]	0.36 ± 0.17[e]

[a]2×10^6 cells were seeded per 25 cm^2 T-flask. The cells were cultivated for 22 hr in 10% FCS-MEM containing BrdUrd (2μg/ml) in the presence of chemicals.
[b]5-Bromo-2′-deoxyuridine.
[c]Mitomycin-C.
[d]Significantly different from controls, $p < 0.001$.
[e]Significantly different from controls, $p < 0.01$.

Table 3 shows the results on sister chromatid exchanges (SCE) induced by MEHP in Chinese hamster cells. An assay was conducted according to the method of Perry and Wolff (*11*). As shown in Table 3, the cells exposed to 5-bromo-2′-deoxyuridine (BrdUrd) for 24 hr produced 5.0 SCE/cell. The cells treated with 25-50 μg of MEHP/ml for 24 hr gave 7.3 and 10.5 SCE/cell, respectively, values which were significantly higher than the numbers of SCE (5.0) obtained with BrdUrd only. When the cells were treated with 750-1500 μg of MEHP/ml for 3 hr, however, only 1500 μg of MEHP/ml produced a significant increase of SCE/cell. MMC, which was used as a positive reference compound, produced 29.5 and 8.8 SCE/cell on the exposure for 24 hr and 3 hr, respectively.

The study by the *in vivo/in vitro* combination assay system with Syrian golden hamster was conducted according to the method developed by Inui (*12*). As shown in Figure 2, 0.5 ml of DMSO containing DEHP or MEHP was administered orally to gravid Syrian golden hamsters on the 11th day of gestation and the animals were kept for 24 hr with free access of laboratory chow and water. The fetuses were then excised and used for primary culture for 3 days. The cells (5×10^5) were then transferred to dishes containing 8-azaguanine (8AG) or 6-thioguanine (6TG) in Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS) in order to examine the induction of the *HGPRT*$^-$ forward mutation. The medium was changed every 24 hr for the first three days and then once every 72 hr for the remaining time.

After a total cultivation period of 15 to 20 days, the cultures were fixed in methanol and stained with Giemsa solution. The number of colonies growing in the medium was counted. Table 4 summarizes the data on chromosomal aberrations induced by the transplacental application of DEHP and MEHP to the embryonic cells of the Syrian golden hamster. The range of the dosage tested was from 3.75 g to 15 g/kg in DEHP and from 0.375 g to 1.5 g/kg in the case of MEHP. As seen in Table 4, 3% of the cells were aberrant in the control experiment, whereas from 10% to 24% of the cells were aberrant with DEHP or MEHP. The types of aberrations include a single chromatid gap, isochromatid gap, a single chromatid break, isochromatid break, chromatid exchanges and other aberrations including chromosome fragmentations and duplications, etc. The morphological transformations of the embryonic cells of the Syrian golden hamster are shown in Table 5.

Cells (5×10^3) of the hamster embryos receiving DEHP or MEHP transplacentally were cultured in 20% FCS-MEM for 7-8 days in plastic dishes (60 mm in diameter) without changing the medium. After fixing and staining with Giemsa solution, the total number of colonies, the number of transformed colonies and the types of colonies were examined. The types of colonies were classified according to Heiderberger et al. (*13*). The transformation rate was expressed as the percentage of morhpologically transformed colonies against the numbers of the survived cells. In this experiment, the cells of the hamster embryos receiving *N*-nitrosodimethylamine

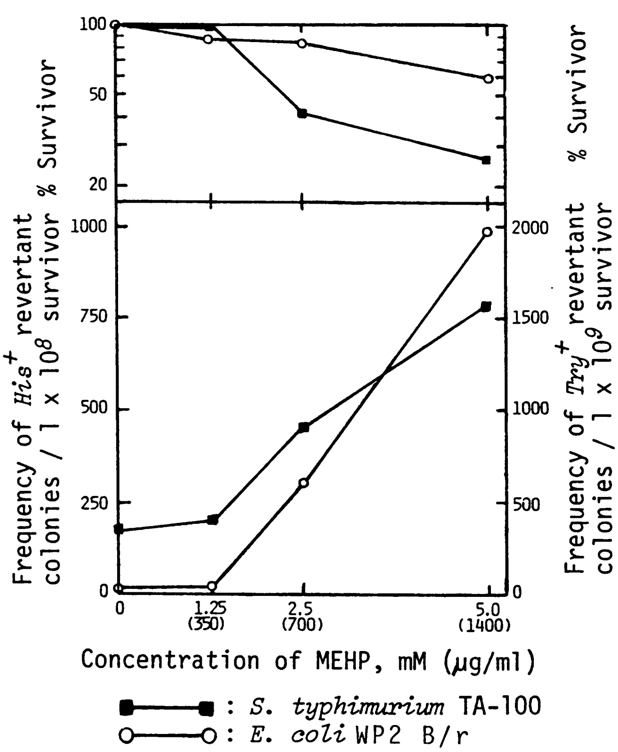

FIGURE 2. Mutagenic activities of MEHP to *S. typhimurium* TA 100 and *E. coli* WP2 Br.

Table 4. Chromosomal aberrations induced by the transplacental application of DEHP and MEHP in embryonic cells of Syrian golden hamster.

Compounds tested	Dose of chemicals, mg/kg	Type of aberration[a] SG	IG	SB	IB	E	Other	Aberrant metaphase cells, %[b]	Normal diploid cells % (range)[c]
DEHP	0	0	0	2	0	0	1	3	55 (28-48)
	3750	3	0	1	2	0	2	8	43 (14-47)
	7500	4	1	3	1	0	5	10[e]	40 (30-70)
	15000	6	3	12	0	4	3	24[f]	70 (30-68)
MEHP	375	6	3	5	0	0	3	14[e]	67 (36-52)
	750	9	1	4	1	0	1	14[e]	66 (35-48)
	1500[d]	5	1	6	2	2	2	22[f]	74 (40-46)

[a]Aberrations: SG, single chromatid gap; IG, isochromatid gap; SB, single chromatid break; IB, isochromatid break; E, exchange; Other, others including ring formation, fragmentation and other chromosomal aberrations.

[b]100 metaphase cells examined.

[c]Percentage of normal diploid cells ($2n = 44$) with show or without chromosomal gaps, breaks or other small abnormalities. The values in parentheses the range of the number of chromosomes.

[d]Only 50 metaphase cells examined.

[e]Significantly different from controls, $p < 0.05$.

[f]Significantly different from controls, $p < 0.001$.

(NDMA) transplacentally were used as a positive control.

As seen from Table 5, the transformation ratio was significantly high in the cells treated with 7500 or 15,000 mg of DEHP/kg and with 375 or 750 mg of MEHP/kg of the body weight of the Syrian golden hamster. Figure 3 shows the microscopic appearance of the cells of morphologically transformed colonies. Figure 3*a* shows normal colonies; Figures 3*b* and 3*c* represent typical transformed colonies of Type II and Type III, respectively, induced by DEHP treatment. Figure 3*d*, which is an enlargement of Figure 3*c*, shows a criss-cross and dense piling up in the center of the cells.

Discussion

The results of our present investigation suggest that DEHP itself is not a mutagen but becomes a mutagen of base pair substitution type after it is hydrolyzed into MEHP. The mutagenic activity of MEHP which was observed in both Rec-assay and reversion plate assay were increased in a dose-related manner. It must be noted, however, that the demonstration of the mutagenicity of MEHP can only be possible within a high and narrow range of its concentration because MEHP has a sterilizing effect at high concentrations and shows no mutagenic activity at low concentrations. Moreover, the activity of MEHP can be completely destroyed by incubating

Table 5. Morphological transformation in embryonic cells of Syrian golden hamster induced by the transplacental application of DEHP and MEHP.

Compounds administered	Dose of chemicals, mg/kg	No. of animals used	Total observed colonies	Transformed colonies	Type of colony[a]		Transformation rate, %[b]
					II	III	
DEHP	0	6	4359	20	11	9	0.46
	3750	5	2557	20	15	5	0.78
	7500	7	3155	49	21	28	1.55[c]
	15000	7	2549	46	22	24	1.80[c]
MEHP	375	5	2537	27	15	12	1.06[d]
	750	6	1913	24	13	11	1.25[e]
	1500	6	1463	13	7	6	0.89
NDMA[f]	200	1	364	14	9	5	3.85[c]

[a]Colony types classified according to Heiderberger et al. (*13*).
[b]Transformation rate is expressed as the percentage of morphologically transformed colonies against the total survivors.
[c]Significantly different from controls, $p < 0.001$.
[d]Significantly different from controls, $p < 0.05$.
[e]Significantly different from controls, $p < 0.01$.
[f]*N*-Nitrosodimethylamine.

Table 6. Summary of the mutagenic and carcinogenic potency of DEHP and MEHP.

Method of testing	Results	
	DEHP	MEHP
Bacterial systems (*in vitro*)		
Rec-assay		
B. subtilis H17, M45	– (+)[a]	+
Reverse mutation		
E. coli WP2	–	+
S. typhimurium TA 98	–	–
S. typhimurium TA 100	– (+)[b]	+ (–)[b]
Mammalian systems		
Direct application (*in vitro*), V79 cells of Chinese hamster		
8AG/6TG-resistant mutation	NP[c]	± (±)[b]
Chromosomal aberration	NP	+
Sister chromatid exchange	NP	+
Transplacental application (*in vivo*/*in vitro*) to embryonic cells of Syrian golden hamster		
8AG/6TG-resistant mutation	±	±
Chromosomal aberration	+	+
Morphological transformation (colony assay)	+	+

[a]Mouse pancreas homogenate was used for the determination of mutagenicity.
[b]Rat liver microsome fraction was used for the determination of mutagenicity.
[c]Not performed.

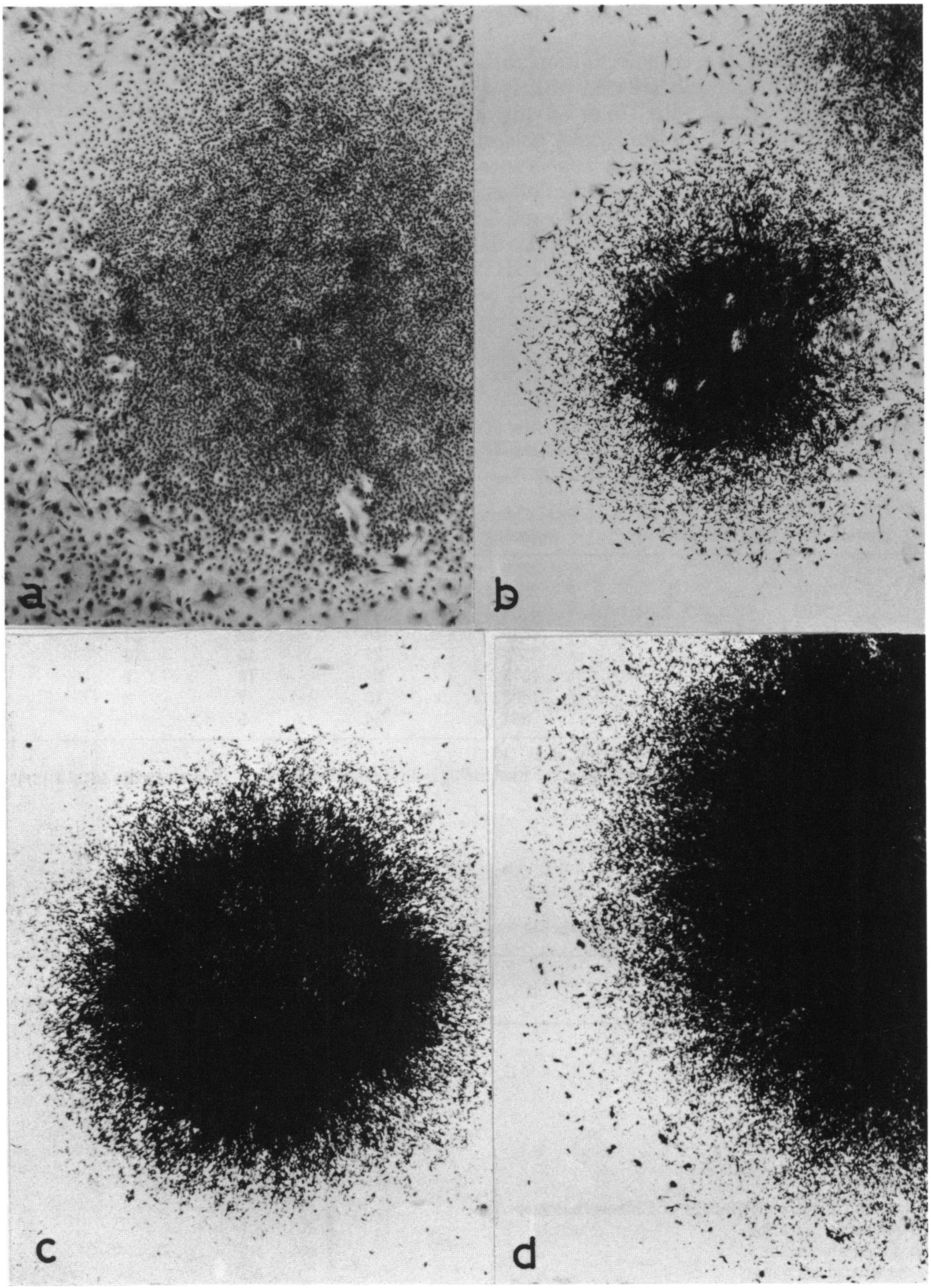

FIGURE 3. Miscroscopic appearance of the morphologically transformed colonies of the embryonic cells from Syrian golden hamster. (a) A normal colony obtained from the culture of DEHP treated cells. The colony appears normal and flat, and the cells have a regular arrangement. (*b*, *c*) Typical transformed colonies of types II and III, respectively, obtained from the culture of DEHP treated cells. In (*b*), the colony consists of short settled fibroplastic cells with massive piling up into virtually opaque multilayers and the cells are only moderately polar. In (*c*), the colony is composed of highly polar, fibroplastic multilayered criss-crossed arrays of densely stained cells. (*d*) Enlargement of Fig. 3*c*.

it with the S9 mix. It is, possibly, for this reason, that the mutagenesis of DEHP (with the S9 mix) and MEHP have not always been demonstrated. As shown in Table 3, SCEs in Chinese hamster V79 cells were induced by MEHP, even at the level of 25 μg/ml in the 24 hr incubation study. As the induction of SCE is believed to be a sensitive reflection of the toxicities related to DNA repair (*14*), it is conceivable that MEHP is the substance having the DNA-damaging effect in Rec-assay and induces SCEs. It was also demonstrated that MEHP induced 8AG- or 6TG-resistant gene mutations and chromosomal aberrations in Chinese hamster V79 cells *in vitro*, though the details of the results are not included in this paper. The effects of DEHP on V79 Chinese hamster cells have not been studied in our laboratory, but negative results will probably be observed unless the conditions are so chosen as to yield enough MEHP during the incubation.

As for the genotoxic effects of DEHP on the embryonic cells of Syrian golden hamster, it will be reasonable to assume that the effects will be due to MEHP formed *in vivo* in the hamster. In fact, 3.9 μg of MEHP/g of fetuses was formed when 10 ml of DEHP/kg was administered orally to gravid Syrian golden hamster at 11-12 days of gestation (our unpublished results). Among several effects of DEHP and MEHP on the embryonic cells of Syrian golden hamster, the positive results on the morphological transformation should be carefully considered, since the cell transformation is closely correlated with carcinogenicity (*13*). The results of our studies on DEHP and MEHP mutagenesis/carcinogenesis are summarized in Table 6.

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